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Award Number: DAMD17-02-1-0452

TITLE: Cyclin E, a Powerful Predictor of Survival in Breast Cancer - A Prospective Study

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REPORT DATE: June 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20040213 036

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual (1 Jun 2002 - 31 May 2003)

4. TITLE AND SUBTITLE

Cyclin E, a Powerful Predictor of Survival in Breast Cancer - A Prospective Study

5. FUNDING NUMBERS

DAMD17-02-1-0452

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Aberrations in expression of cyclin E, a positive cell cycle regulator at the G1 to S transition, may affect the biological behavior of breast cancer. In a retrospective analysis of tumor specimens from 395 breast cancer patients, we found that overexpression of the full-length cyclin E protein and its LMW isoforms was a very strong predictor of breast cancer death regardless of whether patients had lymph node-negative disease or lymph node-positive disease ($p < 0.0001$). Our hypothesis is that dysregulation of cyclin E is a powerful prognostic indicator of outcome in early stage breast cancer. Specifically we will: 1: use cyclin E antibody as a prognostic marker for stage I and II breast cancer in a PROSPECTIVE study, 2: examine the cyclin E associated activity and its immune-complex formation with key cell cycle regulators in freshly resected tumor samples, and 3: develop an immunohistochemical (IHC) assay for specifically detecting the LMW forms of cyclin E in Breast Cancer. We will correlate the cyclin E alterations in samples with the expression of key cell cycle regulators and clinical biomarkers. If we prospectively confirm cyclin E overexpression correlates with poor outcome, clinicians can more appropriately tailor aggressive systemic treatment to those at greatest risk for systemic metastases

14. SUBJECT TERMS

Prospective study, cell cycle, cyclin E, low molecular weight

15. NUMBER OF PAGES

12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

The overall purpose of this 3 year study is to use the altered expression of cyclin E as a prognostic marke for breast cancer.

Despite significant recent advances in understanding the cell biology and molecular genetics of breast cancer, patient prognosis continues to be primarily determined by the pathological extent of disease in the axillary lymph nodes [1]. However, the prognostic information gained from lymph node status is imprecise. For example, over 1/3 of women with lymph node-negative breast cancer who do not receive systemic treatment develop recurrence of their disease within10 years [2, 3]. In addition, approximately 1/3 of patients with positive lymph nodes will be free of recurrence 10 years after local-regional therapy alone [2, 3]. These data highlight the need for more sensitive and specific prognostic factors. Such factors would more precisely inform patients and physicians about expected outcomes and allow for better individual tailoring of systemic treatment.

A number of biological factors have been studied over the past two decades in an attempt to further refine risk categories of breast cancer. Our efforts to find important biological markers in breast cancer have focused on the G1/S checkpoint and how deregulation of this critical phase of the cell cycle affects the virulence and metastatic potential of breast cancer [4-8]. We have specifically been interested in cyclin E because of its importance in the regulation of G1 to S transition in normal dividing cells [9, 10]. In normal dividing cells, the expression of cyclin E regulates the G1 to S transition [9, 10]. Functional knockout of cyclin E by injection of anti-cyclin E antibodies into normal fibroblast cells causes cell arrest in the G1 phase [11]. Conversely, the overexpression of cyclin E protein causes acceleration through G1 along with a

decreased cell size [11, 12]. Due to the crucial role that normal expression and activity of cyclin E plays in cell proliferation, any defects in its expression could have a critical effect in oncogenesis.

Cyclin E gene is amplified in some breast cancer cell lines [6, 13] and we have shown that this amplification can result in as much as 64-fold overexpression of cyclin E mRNA that is constitutively expressed across all phases of the cell cycle [8, 14] (Appendix-Ref 1). Examination of the oncogenic potential of cyclin E in transgenic mice under the control of the bovine ß-lactoglobulin promoter, also revealed that lactating mammary glands of the transgenic mice overexpressing cyclin E, contained hyperplasia and over 10% also developed mammary carcinomas (15). Lastly, constitutive overexpression of cyclin E (but not cyclin D1 or A) in both immortalized rat embryo fibroblasts and human breast epithelial cells results in chromosome instability [15]. Collectively these data provide strong support for the role of cyclin E in breast cancer tumorigenesis.

Several reports have documented a strong correlation between cyclin E overexpression and lack of estrogen receptor expression [5, 16, 17]. More important, we and others have found that high cyclin E levels were associated with a significantly increased risk of death and/or relapse from breast cancer [7, 17, 18], although some studies did not show an association [18, 19].

We discovered that some breast cancer cell lines and human breast cancers can express up to 5 lower molecular weight (LMW) isoforms of cyclin E (ranging in size from 34 to 49 kDa), in addition to overexpression of the full-length cyclin E 50 kDa protein [6, 7, 17, 20]. We hypothesized that these LMW isoforms are active and that the expression of these isoforms correlates with the stage and prognosis of breast cancer [7, 17, 21, 22]. The previous studies that have examined the prognostic importance of cyclin E in breast cancer have used immunohistochemistry (IHC) staining to detect alteration in cyclin E expression [18, 19]. The

antibodies used in these studies cannot distinguish between the form of cyclin E found in both normal and tumor cells and those forms of the protein that are unique to tumor cells.

We hypothesized that these LMW isoforms are active and that the expression of these isoforms correlates with the stage and prognosis of breast cancer [7, 17, 21, 22]. To test this hypothesis, we have recently evaluated the clinical importance of LMW forms of cyclin E in breast cancer in a retrospective study. Using western blot analysis, we measured the expression levels of cyclin E in a cohort of 395 women with primary tumors, and correlated cyclin E expression with other established prognostic factors and clinical outcome. We found cyclin E to be the most powerful independent predictor for survival in stage I-III breast cancer .

Tumor tissue from 395 patients with all stages of breast cancer were used in this study. In addition to western blot (western blot) assay for detection of cyclin E, IHC analysis for cyclin E was also performed in a subgroup of 256 patients, again using an antibody against the carboxy terminus that, on western blot assays, recognizes the LMW forms. On western blot analysis, cyclin E and its LMW isoforms appear as distinct bands; therefore, in order to assess for the prognostic significance of the LMW forms independently, the expression of cyclin E by western blot assay was scored as expression of the full length protein, the LMW isoforms and total cyclin E expression (LMW plus full length). We also examined other clinical and pathologic factors frequently utilized to assess prognosis. These included tumor size, nodal status, clinical stage, her-2/neu expression, DNA ploidy, proliferative index, ER and PR expression, and cyclin D_1 and D_3 levels.

Our results indicated that cyclin E levels, measured by western blot, but not by IHC, had powerful prognostic significance. Overall, disease specific survival was found to be markedly better in patients whose tumors expressed low-negative levels of the cyclin E protein (Figure 1). The prognostic significance of cyclin E was particularly striking in patients with Stage I disease, where only the 12 patients (of 114) whose tumors overexpressed cyclin E died of disease with a

median time to death of 4.1 years. Conversely, as expected, cyclin E levels had no prognostic significance in Stage IV patients.

In a univariate analysis, the variables found to have significant impact on overall survival were patient age, clinical stage, ER/PR status, proliferative index, ploidy, her-2/neu, cyclin D_1, cyclin D_3, full length cyclin E, LMW cyclin E and total cyclin E levels as measured by western blot as well as IHC determined cyclin E level. However, in the multivariate analysis, only nodal status, clinical stage III-IV, ER status and western blot determinations of LMW cyclin E and total cyclin E level retained their significance for predicting death from breast cancer. Using a Cox- proportional hazards model, total cyclin E levels measured by western blot assay remained the most important predictor of death from breast cancer with a hazard ratio of 13.3 compared to a hazard ratio of 2.1 for high levels of LMW cyclin E and a hazard ratio of 1.8 for positive nodes.

Since IHC determination of cyclin E expression was much less useful as a prognostic marker in our patient cohort, we next compared the concordance of IHC versus western blot for measurement of cyclin E levels. We found that 17.5% (21/120) of patients with negative cyclin E by IHC, in fact had elevated cyclin E levels on western blot assay and these patients had poor outcome. Conversely, in over half the patients (74/136) with elevated cyclin E on IHC, western blot analysis revealed only low-normal expression of the protein and these patients in fact had an excellent prognosis.

In summary, our retrospective data supports the hypothesis that tumor specific molecular markers may be important measures of prognosis in cancer patients, improving the sensitivity of current clinical and pathologic based staging systems. Specifically in breast cancer, cyclin E appears to be an important molecular marker that, when compared to clinical stage, tumor size and nodal status, is more accurate for stratification of patients' risk of disease relapse and death. If validated prospectively (the goal of this IDEA award), measures of cyclin E and

other molecular based staging systems will be important determinants of therapy recommendations, sparing many patients toxicity of treatment with little benefit. Our data also highlights the limitations of IHC for determination of cyclin E, although the reasons for this remain unclear. Whether similar false negative and false positive rates are applicable to other molecular markers measured by IHC is unknown. However, this potential pitfall of current IHC techniques is important to consider as more data regarding the clinical utility of molecular markers becomes available.

Results

The scope of our Statement of Work for the first 12 months of the study was to initiate Aim 1:

Aim 1: To use cyclin E antibody as a prognostic marker for stage I and II breast cancer in a PROSPECTIVE study (months 1-36)

A. Freshly resected breast tissue samples (normal adjacent and tumor) from 260 patients diagnosed with stage I and Stage II breast tumors will be collected; RNA, DNA and protein extracted. (months 1-24).

During the first 12 months of the study we have already collected breast tissue samples from 130 patients, prepared whole cell extracts from the biopsy material and subjected them to western blot analysis as described below:

0.1 gram of each matched tissue (normal adjacent tissue and breast cancer tissue obtained from the same patient) were obtained within 30-45 minutes after excision of the tumor. We extracted

protein homogenates from all samples. For whole cell lysate preparation, the tissue specimen were added to one volume of sonication buffer containing a cocktail of protease and phosphatase inhibitors in a low salt buffer, minced and homogenized in a micro-mincer and sonicated at 4°C using a cup-horn adapter to eliminate probe intrusion. Homogenates were then centrifuged at 100,000 X g for 45 minutes at 4°C. The supernatants were aliquoted, and stored at -70°C and subjected to western blot analysis as described [7, 23]. The protein extracts from the 130 tumor specimen (and their corresponding normal adjacent tissue) collected thus far were subjected to Western blot analysis and the expression of cyclin E is being compared and correlated with other known prognostic markers examined in the same samples. We have also examined the expression of other key cell cycle regulators such as cyclin D1, cyclin D3, CDK2, p21, p27, and cyclin A in all the samples.

On the clinical side our research nurse, Mary-Alice Hassett has been working with your office to have our institutionally approved IRB on this study to be approved by the Army. This has taken us a bit longer than anticipated, however it seems that we are in the final stages of the revisions of the Army protocol. Ms. Hassett is also collecting the information on all the clinical biomarkers such as demgraphic data, steroid receptor status, T-stage, N-stage, combined clinical stage.

Conclusions/Future Goal

We have completed our goals for the first 12months of this study which was to accrue 130 patients and subject the lysates to western blot analysis with cyclin E and other biomarkers

Our goal for the coming year is, bar any complications in accruing patients, we should complete our accrual of the additional 130 patients, extract lysates, subject them to western blot analysis and also kinase assays.

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